Ameliorative Effect of Aqueous Leave Extract of *Ocimum Basilicum* on Ccl$_4$ - Induced Hepatotoxicity and Apoptosis in Albino Rats

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**Abstract:** Chemical-induced liver injury depends mostly on the oxidative stress. Basil or sweet basil (*Ocimum basilicum*) is known to have numerous pharmacological activities. The present study aims to investigate the effect of basil on Ccl$_4$-induced hepatotoxicity and apoptotic in albino rats. The result showed CCl$_4$ caused impairment of the normal structural organization of the hepatic lobules, congestion and dilatation of blood vessels, cytoplasmic vacuolization of the hepatocytes, leucocytic infiltrations and fatty degeneration. The biochemical results showed that there was an increase in serum level of ALT, AST, ALP, cholesterol, triglyceride, LDL and HDL. Moreover, CCl$_4$ induced hepatic apoptosis. Treating animals with CCl$_4$ and aqueous leaves extract of *O. basilica* led to an improvement, in both histopathological and biochemical alterations induced by CCl$_4$. Also, apoptosis was repaired by shared administration with both *O. basilicum* and CCl$_4$. These results proved that *O. basilica* had an ameliorative effect against liver injury produced by CCl$_4$ due to its antioxidant activity.

[1. Introduction:]

Oxidative stress has been shown to play a very crucial role in some diseases including liver disease. Free radical that generate inside the body is responsible for oxidative stress and compounds that can scavenge free radicals have great potential in ameliorating these disease processes (Sies, 1997). Basil or sweet basil (*Ocimum basilicum*) is a plant that belongs to the family Labiatae and is known as Tulsi in Hindi, Holy Basil in English and Rehan in Egypt. It is known to have numerous pharmacological activities. Many studies have established that basil leaves extracts have potent antioxidant, anti-aging, anticancer, antiviral, and antimicrobial properties (Chiang et al., 2005; Bozin et al., 2006; Manosroi et al., 2006; Almeida et al., 2007, Akujobi et al., 2010). Orafidiya et al. (2006) investigated the efficacy of the leaf essential oil of *Ocimum gratissimum* Linn. in promoting hair growth in cyclophosphamide-induced hair loss. The results showed that ocimum oil may be capable of enhancing normal hair growth and promoting follicular proliferation in cyclophosphamide-induced hair loss. Sethi (2003) reported that leaves of *Ocimum sanctum* possess good antioxidant as well as antistress potentials in experimental animals. Consumption of basil or basil oil has been associated with a reduction in total cholesterol, low-density lipoprotein and triglyceride (Hicham et al., 2009). Batra and Gupta (2006) indicated that *Ocimum sanctum* leaf supplementation reduced the severity of hydopericardium, hepatitis, myocarditis accompanied with haemorrhages, oedema in lungs, lymphocytic depletion in lymphoid organs and focal interstitial nephritis. Rupert (2009) reported that basil or basil oil have agents for prevention and treatment of cardiovascular disease. It has also been shown that OS leaf extracts can protect the liver from heavy metals (Sharma et al., 2002) and prevent isoproterenel induced myocardial necrosis in rats (Sood et al., 2005).

Chemical-induced liver injury depends mostly on the oxidative stress in hepatic tissue. Carbon tetrachloride (CCl4)-induced liver damage is the best characterized system of xenobiotic-induced hepatotoxicity and is a commonly screening model to evaluate the hepatoprotective potential of drugs with antioxidant properties. Administration of CCl$_4$ causes extensive changes in liver morphology including steatosis, inflammation and necrosis (Qiu et al., 2005). It induced liver fibrosis, cirrhosis, enhanced lipid peroxidation, increases ALT and causes collagen deposition in liver tissue (Nan et al., 2002, Campo et al., 2004). SuYanga et al. (2008) reported that single oral dose of CCl$_4$ produced significantly elevated levels of serum ALT, AST activities and extensive liver necrosis and fatty changes .Carbon tetrachloride was metabolized in
the liver by cytochrome P450 of the endoplasmic reticulum with the formation of a highly toxic trichloromethy radical (CCl3) (Conner et al., 1990).

2. Material and Methods

Animals

Male albino Wistar rats weighing 100 ± 5 g were kept in the laboratory under constant conditions of temperature (24 ± 2 °C) for at least one week before and through the experimental work, being maintained on a standard diet composed of composed of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminized starch. Water was available ad-libitum.

Preparation of ocimum extract

Fresh leaves of Ocimum basilicum were collected from a garden within Genetic Engineering and Biotechnology Research Institute, Menoufia University, Sadat City, Egypt. The leaves were rinsed and Biotechnology Research Institute, Menoufia University, Sadat City, Egypt. The leaves were rinsed with clean water to remove any foreign matter. Leaves were blended with distilled water. The mixture was strained, the merc pressed and the aqueous mixture was filtrated using filter paper. The aqueous extract was used at a dose level of 20 ml/kg O. basilicum (Offiah and Chikwendu, 1999).

Experimental design

All the experiments were done in compliance with the guide for the care and use of laboratory animals. Animals were divided into four groups:

Group 1. Animals were fed on the standard diet and were served as control group.

Group 2. Animals of this group were administrated with oral aqueous O. basilicum extract at a dose level of 20 ml/kg twice a week for 6 weeks.

Group 3. Rats were injected intraperitonealy with 1.0 ml/kg b.w of 10% CCl4 dissolved in olive oil twice a week for 6 weeks (Sakr et al., 2010).

Group 4. Rats were injected with CCl4 (1.0 ml/kg) followed by oral administration with aqueous O. basilicum extract at a dose level of 20 ml/kg twice a week for 6 weeks.

Histological examination

The treated animals and their controls were sacrificed by decapitation after 2, 4 and 6 weeks of treatment. Liver was removed and fixed in Bouin's fluid. Fixed materials were embedded in paraffin wax and sections of 5 micrometres thickness were cut. Slides were stained with haematoxylin and eosin for histological examination.

Biochemical assays

For biochemical assays blood was collected and centrifuged at 3000 rpm for 10 minutes and stored at -20 °C. Liver function enzymes ALT and AST were determined in serum according to the method of Gella et al. (1985). The activity of alkaline phosphatase was assayed by the method of El-Aaeser and El-Marzabani (1975). Cholesterol and triglycerides were measured using the methods of Zlaktis et al. (1953), and Fassati and Prencipe (1982), respectively.

DNA Fragmentation assay

As a measure of apoptotic DNA fragmentation, the presence of DNA ladder was determined according to Wlodek et al. (1991). Extraction of DNA was done according to method of Hassab El-Nabi (2009) and Aljanabi S. M. (1997). 10 mg of liver tissue in eppendorf tubes were lysed with 600 microlitre buffer (50 mM NaCl, 1 mM Na3-EDTA, 0.5% SDS, PH 8.3) and gently shaked. The mixture was incubated overnight at 37 °C then, 20 microlitre of saturated NaCl was added the sample, shacked and centrifuged at 12,000 rpm for 10 min. the supernatant was transferred to new Eppendorf tubes and then DNA precipitated by 600 microlitre cold isoprpranol. The mix was inverted several times till fine fibers appear, and then centrifuged for 5 min. at 12,000 rpm. The supernatant is removed and the pellets were washed with 500 microlitre 70% ethyl alcohol centrifuged at 12,000 rpm for 5 min. After centrifugation the alcohol was decanted or tipped out and the tubes plotted on Whatman paper to be dry. The pellets were resuspended in 50 microlitre or appropriate volume of TE buffer (10 mM Tris, 1 mM EDTA, PH 8). The resuspended DNA was incubated for 30 - 60 min with loading mix (Rnase + loading buffer) and then loaded into the gel wells.

Agarose gel electrophoresis

A gel was prepared with 2% agarose containing 0.1% ethidium bromide (200 ug/ml). The DNA samples were mixed with loading buffer (0.25% bromophenol blue, 0.25% xylene cyanole FF and 30% glycerol) and loaded into the wells (2 ug of DNA/lane) with a standard molecular- sized ladder marker (Pharmacia Biotech., USA). The gel was electrophoresed at a current of 50 mA for 2.5 h using the submarine gel electrophoresis machine. The DNA was visualized and photographed with illumination under UV light using a photodocumentation hood (Fisher Scientific, Pittsburgh, PA, USA) equipped with a Polaroid 667 film with an orange filter (Kodak, Rochester, NY, USA).
Statistical analysis
The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by ANOVA followed by Student’s “t” test using Minitab 12 computer program (Minitab Inc., State Collage, P.A).

3. Results
Histological observations
Figure (1) showed the histological structure of the liver of control rat. Liver of animals administered with O. basilicum appeared with normal structure.

On the other hand, Liver of rats treated with CCl4 for two weeks showed impairment of the normal structural organization of the hepatic lobules and sinusoidal spaces were enlarged. Intrahepatic veins, central and portal were dilated and congested (Fig. 2). Leuocytic infiltrations were observed (Fig.3). Liver sections prepared from rats 4 weeks post-treatment with CCl4 revealed that a considerable number of hepatic cells were damaged and lost their characteristic appearance while others showed marked cytoplasmic vacuolization which was so extensive in some cells to the extent that only slight remnants of the cytoplasmic mass cells - frequently forming a narrow peripheral rim was left (Fig.4). The nuclei of these cells were pyknotic. In addition, congestion of the intrahepatic blood vessels and inflammatory leucocytic infiltrations were observed. The histopathological changes of the liver were more increased after 6 weeks and the liver cells were degenerated and suffered from micro and macrovesicular steatosis (Fig.5). Examination of liver sections obtained from rats treated with both CCl4 and O. basilicum for 2 and 4 weeks revealed gradual restoration of the normal structure of liver tissues. A rare leucocytic infiltration was observed, but the central as well as the portal veins were congested (Fig.6). A large number of binucleated hepatocytes were observed. After 6 weeks, the liver tissue appeared normal and fatty infiltrations was absent in the examined specimens (Fig.7).

Biochemical results
Treatment with CCl4 for 6 weeks caused a highly significant elevation (P<0.05) in the activity of ALT, AST and ALP as compared to those of the control animals. All these parameters were restored to near normal values in rats treated CCl4 and O. basilicum (Figs.8-10). Both control and animals given O. basilicum showed no significant differences in serum activity of ALT, AST and ALP. Administration of CCl4 to rats caused significant increase in cholesterol and triglycerides compared with animals of control groups. Animals treated with both CCl4 and O. basilicum extract showed reduction in their sera level of cholesterol and triglycerides in comparison of those given CCl4 (Figs.11&12). Figures 13 and 14 showed that treating animals with CCl4 induced significant increase in serum HDL and LDL concentrations after 4 and 6 weeks post -treatment compared with control group. On the other hand, animals treated with CCl4 and O. basilicum extract had a noticeable increase in the concentration of these parameters compared with animals received CCl4 alone.

Biochemical features of apoptosis
Administration of carbon tetrachloride for 6 weeks induced fragmentation of DNA in rat livers (Fig.15). The total optical density of released DNA was 128 when compared with controls (table 1). Animals treated with O. basilicum were not display any increased in fragmented DNA and the total optical density was in normal range. Fragmentation of DNA was repaired by shared administration with both O. basilicum and carbon tetrachloride for 6 weeks as rosemary significantly decreased the total optical density of released DNA with value of 25 when compared with CCl4 treated group.

4. Discussion
Results obtained in the present work indicated that CCl4 induced histological and biochemical alterations in liver of albino rats. Concerning the histological effects, liver of CCl4-treated animals showed many degenerative changes including cytoplasmic vacuolization of the hepatocytes, fatty infiltrations, leucocytic infiltrations, congestion of blood vessels, and fibrosis. Similar results were obtained by Sakr et al. (2010) in albino rats intoxicated with CCl4 Moreover, the current results are in accordance with those of Sreelatha et al.(2009) and Lodhi et al.(2009) who reported that liver injury including marked alteration of the entire liver structures with degenerative changes were observed after CCl4 administration. Fatty infiltrations were observed in liver of CCl4 treated rats. In agreement with this result Qiu et al. (2005) and Panovska et al. (2008) reported that CCl4 caused extensive liver necrosis and fatty changes. Brody et al. (1961) attributed the fatty changes in the liver to excessive mobilization of free fatty acids from the fat depots induced by the lipolytic effects of the increased circulating catecholamines and the centrilobular necrosis to the catecholamines-induced decrease in hepatic flow. Liver fibrosis was observed after 6 weeks of treating rats with CCl4. Qiu et al. (2005) reported that CCl4 caused centrilobular necrosis followed by fibrosis. Nan et al.(2002) mentioned that CCl4 is the most widely used chemical for inducing liver fibrosis.
Figs. 1-3: (1) Section of liver of a control rat showing hepatocytes (H), central vein (V), sinusoids (S) and Kupffer cells (K), (2) section of liver of ccl₄-treated rat after two weeks showing congested and enlarged central vein (CV), (3) Showing mass of leucocytic infiltrations (Li), (X 400).

Figs 4-5: (4).Liver section of ccl₄-treated rat for 4 weeks showing cytoplasmic vacuolization of the hepatocytes (arrows),(5). Specimen obtained from a rat treated with ccl₄ for 6 weeks showing fat droplets (fd) of different sizes,(X 400).
Fig 6-7: (6). Liver section of a rat treated with CCl₄ and ocemium for 4 weeks showing congested portal vein (P). (7) Specimen obtained from a rat treated with CCl₄ and ocemium showing an obvious degree of improvement with large number of binucleated cells (arrows), (X 400).

Fig. 8. Change in ALT activity in different experimental groups
Fig. 9. Change in AST activity in different experimental groups

Fig. 10. Change in ALP activity in different experimental groups
Fig. 11. Change in serum cholesterol in different experimental groups

Fig. 12. Change in serum triglycerides in different experimental groups
Fig. 13. Change in HDL in different experimental groups

Fig. 14. Change in LDL in different experimental groups
CCL\textsubscript{4} was found to induce apoptosis as represented by DNA fragmentation. This result came in agreement with Castro et al. (1993) who reported that CCL\textsubscript{4} induced necrosis and DNA fragmentation in Sprague-Dawley male. Rats. Shi et al.(1998) proved that carbon tetrachloride poisoning induced DNA fragmentation, apoptosis and necrosis in rat liver by immunohistochemical labeling of nuclear DNA fragmentation, flow cytometry and gel electrophoresis.

Significant increase in ALT, AST and ALP levels of sera of CCL\textsubscript{4} treated rats was recorded in the present study. In agreement of this result, Wang et al. (1996) observed that a single i.p. injection of CCL\textsubscript{4} caused an increase in ALT and AST. Pablo and Yesenia (2003) found that liver injury induced by CCL\textsubscript{4} in Wistar rats was accompanied by elevation in serum level of ALT,AST and ALP. Increase in triglycerides and cholesterol were recorded in sera after exposure to CCL\textsubscript{4}. Similarly, Torres-Duran et al. (1999) reported that CCL\textsubscript{4} caused elevation in LDL, HDL, triglycerides and cholesterol.

Oxidative stress is a state of redox imbalance caused by increased reactive oxygen species (ROS) generation and decreased antioxidant capacity. Administration of CCL\textsubscript{4} is an established experimental model of severe toxic liver injury involving generation of oxidative stress. It has been reported that exposure to CCL\textsubscript{4} induces oxidative stress in rats (Sharma et al.,1994). Oxidative damage primarily occurs through production of reactive oxygen species, including CCL\textsubscript{3} and CCL\textsubscript{3}O\textsubscript{2} radicals that subsequently react with biological molecules as well as causing damage to membranes (Singh et al.,1998). A decrease in the level of antioxidant enzymes and an increase in lipid peroxidation level were recorded after CCL\textsubscript{4} administration (Campo et al., 2004). The increase in lipid peroxidation in the liver following exposure to CCL\textsubscript{4} may lead to membrane damage resulting in damage of liver cells. The increase in ALT, AST and ALP is the end results of this phenomenon.

The present findings demonstrated that \textit{O.basilicum} improve the histological changes and increased liver function enzyme activity induced by CCL\textsubscript{4}. This indicated the effectiveness of \textit{O.basilicum} in prevention of CCL\textsubscript{4} hepatotoxicity. The hepatoprotective effects of \textit{O.basilicum} have been

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
 & \textbf{Control group.} & \textbf{Ocimum group} & \textbf{CCL4 group} & \textbf{CCL4+ Ocimum group} \\
\hline
\textbf{Intact DNA} & 61 & 58 & 32 & 53 \\
\hline
\textbf{Fragmented DNA} & 5 & 7 & 128 & 25 \\
\hline
\end{tabular}
\caption{Changes in values of the total optical density of both intact and fragmented DNA induced in liver of rat.}
\end{table}

Fig.15. Gel electrophoresis of hepatic DNA M: standard lane, Lane 1: control, lane 2 ocemium, Lane 3: ccl\textsubscript{4} group,, lane 4: ccl\textsubscript{4} and ocemium group.
shown in studies on experimental liver damage. Yamamoto et al., (2005) proved that ocimum suppressed hepatic fibrosis and protected liver against parenchymal damage induced by CCL4. Significant hepatoprotective effects were obtained by ethanolic extract of leaves of O. basilicum against liver damage induced by H2O2 and CCL4 in goat as evidenced by decreased levels of antioxidant enzymes. The extract also showed significant anti lipid peroxidation effects in vitro, besides exhibiting significant activity in superoxide radical and nitric oxide radical scavenging, indicating their potent antioxidant effects (Meera et al., 2009).

Adhvaryu et al., (2007) reported that O. sanctum have hepatoprotective and immunomodulatory effects on liver injury and immunosuppression induced by Isoniazid, Rifampicin and Pyrazinamide in guinea pig. It has been shown that 2% of dried O. sanctum leaf powder supplemented in the diet can lower serum lipid profile and partially protect the liver in diabetic rats (Suanarunsawat and Songsak, 2005). It has also been shown that O. sanctum leaf extracts can protect the liver from heavy metals (Sharma et al., 2002) and prevent isoproterenol induced myocardial necrosis in rats (Sood et al., 2005). O.basilicum treatment attenuated serum lipid profile. This may be due to the anti-hyperlipidemic action of components of O.basilicum leaves. Suanarunsawat et al. (2009) mentioned that the anti-hyperlipidemic activity of O.basilicum may be due to the suppression of liver lipid synthesis. Zhang et al (2009) reported that the main components of O. basilicum are: linalool (29.68%) , (Z)-cinnamic acid methyl ester (21.49%) , cyclohexene (4.41%) , alpha-cadinol (3.99%) , 2,4-dioisopropenyl-1-methyl-1-vinylcyclohexane (2.27%), 3,5-pyridine-dicarboxylic acid, 2,6-dimethyl-diethyl ester (2.01%), beta-cubenene (1.97%), guaia-1(10),11-diene (1.58%), cadinene (1.41%) (E)-cinnamic acid methyl ester (1.36%) and beta-guaiene (1.30%). Lee and Scagel (2009) reported that the presence of chicoric acid (dicaffeoyltartaric acid) was the major phenolic compound, in basil leaves. O.basilicum is rich source of flavonoids which have been shown to possess various biological properties related to antioxidant mechanisms. Dasgupta et al., (2007) reported that O.basilicum increased the activity of xenobiotic metabolizing phase I and phase II enzymes, elevating antioxidant-enzyme response by increasing significantly the hepatic glutathione reductase, superoxide dismutase, and catalase activities, increasing glutathione content and decreasing lipid peroxidation and lactate dehydrogenase activity in the liver of mice. Chinnasamy et al., (2007) reported that the protective action of ocimum was attributed to its antioxidant action. They added that this protection may be also due to anti-inflammatory property of ocimum which reduces formation, release, and activity of inflammatory mediators such as cytokines, histamine, prostaglandins, and leukotrienes.

Suanarunsawat et al. (2009) reported that O. sanctum leaf have lipidlilowering effect and antioxidant activity in rats fed with a high cholesterol diet. It is concluded from the present work that the hepatoprotective of O. basilicum may be attributed to the antioxidant activity of its flavonoids.

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